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(54) Title: COMPOSITIONS AND METHODS FOR THE TREATMENT OF CANCER, SCREENING OF PUTATIVE ANTI-CANCER COMPOUNDS, AND ASSESSING CANCER PROGRESSION

(57) Abstract: The present invention provides novel methods for the treatment of cancer, methods for screening compounds having anti-cancer activity, and methods of assessing cancer progression.

**COMPOSITIONS AND METHODS FOR THE TREATMENT OF CANCER,  
SCREENING OF PUTATIVE ANTI-CANCER COMPOUNDS, AND  
ASSESSING CANCER PROGRESSION**

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10        This invention claims priority under 35 U.S.C.  
§119(e) to US Provisional Application Number 60/427,326  
filed November 18, 2002. The entire disclosure of the  
above-identified application is incorporated by reference  
herein.

15

**FIELD OF THE INVENTION**

      The present invention relates to the fields of  
cellular biology and oncology. Specifically, the present  
invention provides novel methods for the treatment of  
20 cancer, methods for screening compounds having anti-  
cancer activity, and methods of assessing cancer  
progression.

**BACKGROUND OF THE INVENTION**

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      A number of literature and patent references are  
cited in the instant application in order to more fully  
describe the state of the art to which this invention  
pertains. The entire disclosure of each of these  
30 citations is incorporated by reference herein.

      Gastrointestinal stromal tumors (GISTs) are the  
most common mesenchymal malignancies of the GI tract.  
The neoplastic cells in GISTs appear to belong to the  
same lineage as the interstitial cells of Cajal (ICC),  
35 which are the pacemaker cells regulating gastrointestinal  
peristaltic activity. GISTs share many  
immunohistochemical, morphological, and ultrastructural

features with ICCs that support this hypothesis. In a small number of cases, however, these tumors can be located outside the GI tract (omentum, peritoneum, and retroperitoneum) in tissues that are not known to contain ICCs and, therefore, the true progenitor cell is controversial. These GI sarcomas are characterized by the presence of constitutively activated KIT (CD117), the receptor tyrosine kinase (RTK) encoded by the *c-KIT* proto-oncogene, also known as stem cell factor receptor. *c-KIT* is a member of the RTK subclass III family and has structural homology to the receptors for FLT3, platelet-derived growth factor (PDGF) and macrophage colony-stimulating factor. The proposed mechanism of constitutive KIT tyrosine phosphorylation in most GIST is a gain of function mutation in the *c-KIT* gene. These mutations generally involve either missense nucleotide substitutions or in-frame deletions, but certain GISTs have in-frame insertions, resulting in *c-KIT* activation. Most *c-KIT* mutations, in GISTs, involve exon 11, which encodes a portion of the cytoplasmic juxtamembrane domain. Smaller numbers of mutations involve, without limitation, exon 9 (extracellular domain), exon 13 (first part of the split tyrosine kinase domain), or exon 17 (phosphotransferase domain) [Lasota et al., Am. J. Pathol. 154, 53-60 (1999); Rubin et al., Cancer Res. 61, 8118-8121 (2001); Hirota et al., Science 279, 577-580 (1998); Nakahara et al., Gastroenterology 115, 1090-1095 (1998); Lux et al., Am. J. Pathol. 156, 791-795 (2000)].

Most GISTs are diagnosed in middle-aged and older adults and can occur anywhere in the gastrointestinal tract but predominantly are localized to the stomach. Malignant GISTs often present with synchronous metastatic disease and even those amenable to complete surgical

resection often recur locally, with a 5-year actuarial survival rate of approximately 50%. Patients with metastatic disease uniformly have a poor prognosis with a median survival of 6-8 months, as GISTs have been

5 historically resistant to conventional and investigational therapy.

Imatinib (imatinib mesylate, GLIVEC®/GLEEVEC®, Novartis, Basel, Switzerland), formerly known as STI571, is a 2-phenylaminopyrimidine derivative orally

10 administrated drug which in pre-clinical studies inhibits c-Abl, Bcr-Abl, and PDGF receptors [Buchdunger et al., J. Pharmacol. Exp. Ther. 295, 139-145 (2000); Druker, B.J., Trends Mol Med 8, S14-8. (2002)]. Imatinib (N-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-4-(4-

15 methyl-piperazin-1-ylmethyl)-benzamide) is described, e.g., in EP 0 564 409 B1 and, in the form of the methane sulfonate salt, in WO 99/03854. Several phase I/II clinical trials have demonstrated the efficacy of imatinib in the treatment of CML patients with the

20 Philadelphia chromosome and the BCR/ABL translocation [Kantarjian et al., N. Engl. J. Med. 346, 645-652 (2002); Talpaz et al., Blood 98, 845a (2001); Sawyers et al., Blood 99, 3530-9. (2002)]. This drug has also been shown to be a specific *in vitro* inhibitor of c-KIT

25 phosphorylation in several tumor cell lines [Buchdunger et al., J. Pharmacol. Exp. Ther. 295, 139-145 (2000); Heinrich et al., Blood 96, 925-932 (2000)]. After suggestive initial pre-clinical studies it was proposed that activated c-KIT was an early event in the initiation

30 and progression of the malignant phenotype for GIST. A recent report indicated that imatinib rapidly inhibits c-KIT phosphorylation and tumor cell proliferation, while inducing apoptosis, in an established human GIST cell

line (GIST882). GIST882 is an immortal GIST cell line that expresses an activating c-KIT mutation encoded by a homozygous exon 13 missense mutation (i.e., K642E amino acid substitution) [Tuveson et al., Oncogene 20, 5054-5058 (2001)]. The KIT RTK target has been exploited in two recently completed phase I/II clinical trials of GIST. Over 50% of patients with unresectable or metastatic GISTs demonstrated a classic partial response and only 10% of patients manifested disease progression while receiving imatinib [Blanke et al., Prog. Proc. Am. Soc. Clin. Oncol. 21, 1608a (2002); van Oosterom et al., Lancet 358, 1421-1423 (2001)].

Despite its early clinical success there are limited pre-clinical and clinical data on the molecular targets of imatinib downstream from c-KIT. In particular there has not been a comprehensive evaluation of changes in GIST gene expression following imatinib therapy.

#### SUMMARY OF THE INVENTION

In accordance with the present invention, a method of assessing the anti-cancer activity of a KIT tyrosine kinase inhibitor in a biological sample comprising a tumor cell is provided. An exemplary method comprises detecting alterations in expression levels of a nucleic acid sequence provided in Tables 2, 3 and 4 in response to administration the KIT tyrosine kinase inhibitor. The present inventors have identified specific alterations in expression patterns of the sequences listed in Tables 2, 3 and 4 in response to administration of anti-cancer agents. In a preferred embodiment, the tumor is a gastrointestinal stromal tumor and the KIT tyrosine kinase inhibitor is imatinib, SU11248 (Sugen Pharmaceuticals), or a pharmaceutically

acceptable salt thereof. In a particularly preferred embodiment, the method comprises detecting the level of expression of a nucleic acid sequence encoding Sprouty4A protein. The method also encompasses determining  
5 expression levels of the Sprouty4A protein itself using an antibody that specifically binds to the Sprouty4A protein.

Also within the scope of the present invention is a method of screening a compound for activity against a  
10 tumor. An exemplary method comprises contacting mammalian tumor cells with the compound and detecting in said tumor cells an altered level of expression of a nucleic acid listed in at least one table selected from the group consisting of Table 2, Table 3 and Table 4  
15 relative to a control. In a preferred embodiment, compounds are screened using the gastrointestinal stromal tumor cell line, GIST882.

In yet another aspect, a method for determining the efficacy of an anticancer treatment comprising detection  
20 of an alteration in expression levels of a biomarker are provided. Suitable biomarkers for this purpose comprise a nucleic sequence provided in a table selected from the group consisting of Table 2, Table 3, and Table 4. Preferably, the biomarker is sprouty 4, the tumor is GIST  
25 and sprouty 4 expression level is decreased in response to effective anticancer treatment. In an alternative embodiment, the tumor is GIST, the biomarker is MAFbx and MAFbx expression level is elevated in response to effective anticancer treatment.

30 In yet another aspect of the invention, a method for determining the efficacy of an anticancer treatment comprising detection of an alteration in post-translation modification of a biomarker is provided. Again, suitable

nucleic acids for this purpose comprise a nucleic sequence provided in a table selected from the group consisting of Table 2, Table 3, and Table 4. In a preferred embodiment, the tumor is GIST, the biomarker is  
5 GAB1, and the post translational modification is phosphorylation which is decreased in response to effective anti-cancer treatment.

#### BRIEF DESCRIPTIONS OF THE DRAWING

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Figure 1 is an image of a cDNA microarray analysis of SPRY4A (Sprouty4A) expression. Snapshots of actual hybridization signal pairs from spotted SPRY4A cDNA fragment when hybridized with Cy3- and Cy5- labeled cDNA  
15 from GIST cells treated and untreated with 10  $\mu$ M imatinib for 6, 12, 24, and 48 hours. The last pair (C) represents hybridization signals of  $\beta$ -actin in both channels as a control.

20 Figure 2A is a graph of a growth curve of imatinib-treated and untreated control cells. The X axis represents hours of treatment and the Y axis represents the total number of cells ( $\times 10^5$ ). Untreated GIST882 cells (diamonds); GIST882 cells treated with 1  $\mu$ M imatinib  
25 (squares); GIST882 cells treated with 10  $\mu$ M imatinib (triangles). Figure 2B is a schematic alignment of SPRY4A [GenBank Accession No. AF227516 (Homo sapiens Sprouty4A mRNA, complete cDNA); Protein ID: AAK00652.1] and SPRY4C [GenBank Accession No. AF227517 (Homo sapiens  
30 Sprouty4C mRNA, complete cDNA); Protein ID: AAK00653.1]. Diagonally shaded areas represent sequence unique for SPRY4A, vertically shaded area is the sequence unique for SPRY4C, black box represents the fragment of the cDNA

sequence spotted on the microarray, arrows indicate the position of primer sets used for reverse transcription polymerase chain reaction (RT-PCR) analysis. Figure 2C is an image of a Northern blot analysis of SPRY4A expression. In the upper panel, the SPRY4A mRNA levels in imatinib treated and control GIST cells are shown. In the lower panel, an image of the ethidium bromide-stained gel prior to blotting is shown. The position of the 28s and 18s rRNA is indicated at the left of the panels. Figure 2D is an image of a gel representing the RT-PCR analysis of SPRY4 expression. In the upper panel, the isoform determination and the level of the SPRY4 RT-PCR product in imatinib treated and untreated GIST cells are shown. In the lower panel,  $\beta$ -actin RT-PCR product levels in drug treated and untreated GIST cells are shown.

Figure 3A is a series of immunoblots showing the levels of phospho-c-Kit/total c-Kit, phospho-AKT/total AKT, and phospho-ERK1/2/total ERK1/2 in imatinib (1 and 10  $\mu$ M) treated and untreated GIST cells. Figure 3B is an image of an RT-PCR analysis of SPRY4A and  $\beta$ -actin levels in imatinib treated and untreated GIST cells.

Figure 4A is a series of immunoblots showing phospho-ERK1/2/total ERK1/2, phospho-AKT/total AKT levels in U0126, a MEK inhibitor, treated and untreated GIST cells. Figure 4B is an image of an RT-PCR analysis of SPRY4A and  $\beta$ -actin levels in U0126 treated and untreated GIST cells.

Figure 5A is a gel image showing SPRY4A expression in response to imatinib treatment in clinical cases of GIST. In the upper panel, the levels of SPRY4A RT-PCR product is shown before and after drug administration in six



patients with GIST [patients #1, 2, 3 responded favorably to the drug, patient #4 initially responded but subsequently progressed (R-P), patients #5 and 6 failed to respond to treatment (NR), patient #7 had one tumor  
5 respond to treatment and one tumor that did not]. In the lower panel,  $\beta$ -actin RT-PCR product levels are shown as a control. Figure 5B is a gel image showing the RT-PCR analysis of MAFbx for patients 1, 4, 6, and 7. The lower panel is a  $\beta$ -actin control.

10

Figure 6 is a series of immunoblots showing the phospho-c-KIT and total c-KIT protein (Fig. 6A); phospho-AKT and total AKT levels (Fig. 6B); and phospho-ERK1/2 and total ERK1/2 levels (Fig. 6C) in liposarcoma, GIST cells and  
15 imatinib resistant GIST.

Figure 7A is a series of gel images showing the RT-PCR analysis of SPRY1, 2, 3, and 4 in untreated and imatinib treated GIST cells.  $\beta$ -actin is provided as a control.  
20 Figure 7B is a Western blot, employing a SPRY4 specific antibody, of lysates from GIST cells treated with imatinib for 0, 24, 72, and 120 hours.

Figure 8A is a series of gel images of the RT-PCR  
25 analysis of imatinib-responsive genes. RNA was isolated from imatinib-treated (10  $\mu$ M for 6, 12, 24, and 48 h) and untreated GIST882 cells and reverse transcribed. Shown are ethidium bromide stained agarose gels of the RT-PCR products for SPRY4A, MAFbx, FZD8, PDE2A, RTP801,  
30 FLJ20898, and ARHGEF2.  $\beta$ -Actin is shown as a control. Fig. 8B is a series of gel images of the RT-PCR analysis of SPRY4A, MAFbx, FZD8, PDE2A, RTP801, FLJ20898, ARHGEF2, and  $\beta$ -actin levels in U0126-treated and untreated GIST

cells. Fig. 8C a series of gel images of the RT-PCR analysis of SPRY4A, MAFbx, FZD8, PDE2A, RTP801, FLJ20898, ARHGEF2, and  $\beta$ -actin levels in imatinib-treated and untreated GIST cells. Fig. 8D is a series of gel images of the RT-PCR analysis of SPRY4A, MAFbx, and  $\beta$ -actin levels in LY294002-treated and untreated GIST cells.

Figure 9 is a series of Western blots. GIST cells were incubated with imatinib mesylate for 5, 15, 30, 60, and 120 minutes (lanes 1-6, respectively). The cells were lysed and proteins were separated by acrylamide gel electrophoresis and Western blotting was performed using antibodies to GAB1 and phospho-GAB1 (P-GAB1; upper panel) or GAB2 and phospho-GAB2 (P-GAB2; lower panel). Antibodies were obtained from Cell Signaling Technology with the exception of GAB2 which was obtained from Upstate Biotechnology.

#### DETAILED DESCRIPTION OF THE INVENTION

DNA microarrays revealed 148 genes that were differentially expressed between untreated and imatinib-treated human GIST cells, *in vitro*. One of these genes, Sprouty4A (SPRY4A) a regulator of tyrosine kinase-mediated signaling pathways, was dramatically down-regulated (~7-fold) for the entire duration of drug exposure (6-48 h). In addition, imatinib inhibited KIT phosphorylation without affecting the total level of KIT protein. The constitutive activation of extra-cellular signal regulated kinase (ERK) 1/2 and AKT was also inhibited without affecting the total cellular levels of either protein, however, down-regulation of SPRY4A was dependent on the activation of the ERK1/2 pathway and was independent of AKT activation. To investigate the

clinical correlation of these findings GIST specimens were examined from patients on a phase II imatinib treatment trial. Tumor biopsies from patients with non-responding GIST continued to express high levels of SPRY4A, whereas SPRY4A expression was down-regulated in GIST biopsies obtained during imatinib therapy from patients who clinically demonstrated durable partial response. Evaluation of available tissue from one of the non-responder found that high levels of SPRY4A mRNA positively correlated with expression of constitutively active forms of c-KIT and ERK1/2. Furthermore, this tumor possessed a mutation in exon 9 of c-KIT that was associated with a failure to respond to imatinib. This in frame mutation (1530ins6) in exon 9 of c-KIT has previously been described in a study of Japanese GIST patients [Sakurai et al., Jpn J Cancer Res 92, 494-8 (2001)], but has not been correlated with resistance to imatinib.

Sprouty was originally identified as a down-regulator of the "Breathless" (the Drosophila equivalent of fibroblast growth factor receptor) signaling cascade that governs tracheal branching [Hacohen et al., Cell 92, 253-63 (1998)]. Sprouty is an intracellular protein associated with the inner surface of the plasma membrane that binds two intracellular components of the Ras pathway, Drk (the Drosophila homolog of Grb2) and Gap1, a Ras GTPase-activating protein [Casci et al., Cell 96, 655-65 (1999)]. Sprouty is an inhibitor of Ras signal transduction in particular and of RTK signaling in general [Casci et al., Cell 96, 655-65 (1999); Reich et al., Development 126, 4139-47. (1999)]. While Drosophila has only one Sprouty protein, mammals have at least five related proteins (Sprouty 1, 2, 3, 4A, and 4C) encoded by

four genes. The Sprouty proteins are classified under the same gene family by virtue of their characteristic cysteine-rich residues located in their carboxyl termini.

SPRY4A (sometimes referred to herein as SPRY4) may  
5 also have an active role in melanocytes, mastocytes and other c-KIT expressing cells. SPRY4A is constitutively expressed in liposarcomas and leiomyosarcomas (data not shown), sarcoma histotypes that as a group are unresponsive to imatinib. In this respect, it has been  
10 shown that c-KIT is not expressed in these tumors (Fig. 6 and data not shown), but that ERK1/2 is constitutively activated. Therefore, it is possible that SPRY4A expression in these tumors may be regulated via the EGFR signaling pathway given that EGF treatment can also  
15 induce Spry4 expression in mammalian cells [Sasaki et al., J Biol Chem 276, 36804-8 (2001)]. Sprouty has also been demonstrated to interact with Grb2 [Casci et al., Cell 96, 655-65 (1999)] and Grb2, in turn, has been found to be tyrosine phosphorylated by Bcr/Abl [Li et al., Embo  
20 J 20, 6793-804 (2001)]. These findings further implicate Sprouty in the therapeutic response to imatinib seen among CML patients.

As demonstrated herein, expression of SPRY4A is a reliable marker of KIT tyrosine kinase inhibitor response  
25 in GISTs. The findings also point to SPRY4A as a potential genetic endpoint of the KIT tyrosine kinase inhibitor-induced arrest of the c-KIT signaling pathway. This can translate into the clinical application of SPRY4A expression as a tool for early discrimination  
30 between patients that respond to therapy with a KIT tyrosine kinase inhibitor such as imatinib and those who will progress.

The level of expression of a gene whose expression level correlates with the biological activity of a KIT tyrosine kinase inhibitor can be measured by any technical means such as, without limitation, by  
5 monitoring RNA expression by using, for example, the techniques of RT-PCR or Northern blotting; or by monitoring protein expression by using, for example, the techniques of Western blotting, ELISA or immunohistochemistry.

10       Antibodies that specifically bind to the Sprouty4A protein can be produced in accordance with known methods. The Sprouty4A protein or parts thereof can be produced, e.g., by conventional recombinant DNA techniques or, alternatively, be synthesized chemically using standard  
15 peptide synthesis techniques. These proteins/peptides can then be used to generate antibodies directed against the Sprouty4A protein using known immunization methods. In order to generate antibodies which bind to Sprouty4A but not to Sprouty4C or other Sprouty proteins, the  
20 antibodies are preferably raised against peptide sequences which are present in the Sprouty4A isoform but not in the Sprouty4C isoform or other Sprouty proteins, such as, for example, the N-terminal sequence which has minimal homology among the Sprouty family. Antibodies  
25 that specifically bind to the Sprouty4A protein can be used to detect the level of expression of the Sprouty4A protein in a biological sample by means of, e.g., Western blotting, ELISA, or immunohistochemistry.

The general terms used hereinbefore and hereinafter  
30 preferably have the following meanings, if not indicated otherwise:

"Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single

or double stranded and, if single stranded, the molecule of its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated. For example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism.

When applied to RNA, the term "isolated nucleic acid" refers primarily to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from other nucleic acids with which it would be associated in its natural state (i.e., in cells or tissues). An isolated nucleic acid (either DNA or RNA) may further represent a molecule produced directly by biological or synthetic means and separated from other components present during its production.

"Natural allelic variants", "mutants" and "derivatives" of particular sequences of nucleic acids refer to nucleic acid sequences that are closely related to a particular sequence but which may possess, either naturally or by design, changes in sequence or structure. By closely related, it is meant that at least about 75%, but often, more than 90%, of the nucleotides of the

sequence match over the defined length of the nucleic acid sequence. Changes or differences in nucleotide sequence between closely related nucleic acid sequences may represent nucleotide changes in the sequence that  
5 arise during the course of normal replication or duplication in nature of the particular nucleic acid sequence. Other changes may be specifically designed and introduced into the sequence for specific purposes, such as to change an amino acid codon or sequence in a  
10 regulatory region of the nucleic acid. Such specific changes may be made in vitro using a variety of mutagenesis techniques or produced in a host organism placed under particular selection conditions that induce or select for the changes. Such sequence variants  
15 generated specifically may be referred to as "mutants" or "derivatives" of the original sequence.

The term "functional" as used herein implies that the nucleic or amino acid sequence is functional for the recited assay or purpose.

20 A "replicon" is any genetic element, for example, a plasmid, cosmid, bacmid, phage or virus, that is capable of replication largely under its own control. A replicon may be either RNA or DNA and may be single or double stranded.

25 A "vector" is a replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

30 An "expression operon" refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e.g., ATG or AUG

codons), polyadenylation signals, terminators, and the like, and which facilitate the expression of a polypeptide coding sequence in a host cell or organism.

The term "probe" as used herein refers to an  
5 oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences  
10 complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the  
15 complexity of the target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides. The probes herein are selected to be "substantially" complementary to different strands of a particular target nucleic acid  
20 sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact  
25 complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer  
30 sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.



The term "specifically hybridize" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

In accordance with the present invention, nucleic acids having the appropriate sequence homology with the nucleic acids of the invention may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al. (1989, Molecular Cloning, Cold Spring Harbor Laboratory), using a hybridization solution comprising : 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS ; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS ; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS ; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between

nucleic acid molecules of a specified sequence homology (Sambrook et al., 1989, supra):

$$T_m = 81.5^{\circ}\text{C} + 16.6 \log [\text{Na}^+] + 0.41 (\% \text{ G+C}) - 0.63 (\% \text{ formamide}) - 600/\text{\#bp in duplex}$$

As an illustration of the above formula, using  $[\text{Na}^+] = 0.368$  and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the  $T_m$  is 57°C. The  $T_m$  of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C.

The stringency of the hybridization and wash depend primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of annealing of the probe with its target, the hybridization is usually carried out at salt and temperature conditions that are 20-25°C below the calculated  $T_m$  of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be approximately 12-20°C below the  $T_m$  of the hybrid. In regards to the nucleic acids of the current invention, a moderate stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 2X SSC and 0.5% SDS at 55°C for 15 minutes. A high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 1X SSC and 0.5% SDS at 65°C for 15 minutes. A very high stringency hybridization is defined as hybridization in 6X SSC, 5X

Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 0.1X SSC and 0.5% SDS at 65°C for 15 minutes.

The terms "transform", "transfect", "transduce",  
5 shall refer to any method or means by which a nucleic acid is introduced into a cell or host organism and may be used interchangeably to convey the same meaning. Such methods include, but are not limited to, transfection, electroporation, microinjection, PEG-fusion and the like.  
10 The introduced nucleic acid may or may not be integrated (covalently linked) into nucleic acid of the recipient cell or organism. In bacterial, yeast, plant and mammalian cells, for example, the introduced nucleic acid may be maintained as an episomal element or independent  
15 replicon such as a plasmid. Alternatively, the introduced nucleic acid may become integrated into the nucleic acid of the recipient cell or organism and be stably maintained in that cell or organism and further passed on or inherited to progeny cells or organisms of the  
20 recipient cell or organism. In other manners, the introduced nucleic acid may exist in the recipient cell or host organism only transiently.

A "cell line" is a clone of a primary cell or cell population that is capable of stable growth in vitro for  
25 many generations.

Suitable chemotherapeutic agents are any compounds that exhibit anticancer activity including, but are not limited to: alkylating agents (e.g., nitrogen mustards such as chlorambucil, cyclophosphamide, isofamide,  
30 mechlorethamine, melphalan, and uracil mustard; aziridines such as thiotepa; methanesulphonate esters such as busulfan; nitroso ureas such as carmustine, lomustine, and streptozocin; platinum complexes such as

cisplatin and carboplatin; bio-reductive alkylators such as mitomycin, procarbazine, dacarbazine and altretamine); DNA strand-breakage agents (e.g., bleomycin); topoisomerase II inhibitors (e.g., amsacrine,

5 dactinomycin, daunorubicin, idarubicin, mitoxantrone, doxorubicin, etoposide, and teniposide); DNA minor groove binding agents (e.g., plicamycin); antimetabolites (e.g., folate antagonists such as methotrexate and trimetrexate; pyrimidine antagonists such as fluorouracil,

10 fluorodeoxyuridine, CB3717, azacitidine, cytarabine, and floxuridine; purine antagonists such as mercaptopurine, 6-thioguanine, fludarabine, pentostatin; asparaginase; and ribonucleotide reductase inhibitors such as hydroxyurea); tubulin interactive agents (e.g., vincristine,

15 vinblastine, and paclitaxel (Taxol)); hormonal agents (e.g., estrogens; conjugated estrogens; ethinyl estradiol; diethylstilbesterol; chlortrianisen; idenestrol; progestins such as hydroxyprogesterone caproate, medroxyprogesterone, and megestrol; and

20 androgens such as testosterone, testosterone propionate, fluoxymesterone, and methyltestosterone); adrenal corticosteroids (e.g., prednisone, dexamethasone, methylprednisolone, and prednisolone); leutinizing hormone releasing agents or gonadotropin-releasing

25 hormone antagonists (e.g., leuprolide acetate and goserelin acetate); and antihormonal antigens (e.g., tamoxifen, antiandrogen agents such as flutamide; and antiadrenal agents such as mitotane and aminoglutethimide). Preferably, the chemotherapeutic

30 agent is selected from the group consisting of: placitaxel (Taxol®), cisplatin, docetaxol, carboplatin, vincristine, vinblastine, methotrexate, cyclophosphamide, CPT-11, 5-fluorouracil (5-FU), gemcitabine, estramustine,

carmustine, adriamycin (doxorubicin), etoposide, arsenic trioxide, irinotecan, and epothilone derivatives.

The term "KIT tyrosine kinase inhibitor" is understood to mean that in the tyrosine kinase inhibition assay described below (c-Kit Enzyme Assay), such a compound inhibits the tyrosine kinase activity of the c-Kit tyrosine kinase domain by 50% (IC<sub>50</sub>) in a concentration of less than 10  $\mu$ M, preferably less than 1  $\mu$ M, more preferably less than 100 nM, most preferably less than 10 nM. In a broader sense, the term "KIT tyrosine kinase inhibitor" also refers to compounds which decrease KIT tyrosine kinase activity indirectly, i.e., not through direct inhibition of the tyrosine kinase domain of KIT. Such compounds may, for example, decrease the expression levels of KIT, inhibit the pathway that leads to activation of KIT or act as KIT antagonists. Methods to test a compound's ability to exhibit such a KIT inhibitory activity are well known in the art. Preferably, the following is meant by a KIT tyrosine kinase inhibitor: Imatinib, PTK787 [1-(4-chloroanilino)-4-(4-pyridylmethyl)phthalazine; see US 6,258,812], PD180970, ZD4190, SU5416, SU6668 and pharmaceutically acceptable salts of these compounds, especially imatinib and pharmaceutically acceptable salts thereof, most preferably imatinib mesylate (GLIVEC®/GLEEVEC®). Imatinib mesylate can be administered, e.g., in the form as it is marketed under the trademark GLIVEC® or GLEEVEC®.

As an example of a c-Kit Enzyme Assay, the baculovirus donor vector pFbacG01 (GIBCO) may be used to generate a recombinant baculovirus that expresses the amino acid region amino acids 544-976 of the cytoplasmic kinase domain of human c-Kit. The coding sequences for

the cytoplasmic domain of c-Kit may be amplified by PCR from a human uterus c-DNA library (Clontech). The amplified DNA fragment and the pFbacG01 vector may be made compatible for ligation by digestion with BamHI and  
5 EcoRI. Ligation of these DNA fragments results in the baculovirus donor plasmid c-Kit. The production of the viruses, the expression of proteins in Sf9 cells and the purification of the glutathione-S-transferase (GST)-fused proteins may be performed as follows:

10       *Production of virus:* Transfer vector (pFbacG01-c-Kit) containing the c-Kit kinase domain is transfected into the DH10Bac cell line (GIBCO) and the cells are plated on selective agar plates. Colonies without  
15 insertion of the fusion sequence into the viral genome (carried by the bacteria) are blue. Single white colonies are picked and viral DNA (bacmid) is isolated from the bacteria by standard plasmid purification procedures. Sf9 or Sf21 cells (American Type Culture  
20 Collection) are then transfected in 25 cm<sup>2</sup> flasks with the viral DNA using Cellfectin reagent.

*Determination of small scale protein expression in Sf9 cells:* Virus containing media is collected from the transfected cell culture and used for infection to increase its titer. Virus containing media obtained  
25 after two rounds of infection is used for large-scale protein expression. For large-scale protein expression 100 cm<sup>2</sup> round tissue culture plates are seeded with 5 x 10<sup>7</sup> cells/plate and infected with 1 mL of virus-containing media (approx. 5 MOIs). After 3 days the cells are  
30 scraped off the plate and centrifuged at 500 rpm for 5 min. Cell pellets from 10-20, 100 cm<sup>2</sup> plates, are resuspended in 50 mL of ice-cold lysis buffer (25 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1% NP-40, 1 mM DTT, 1 mM

PMSF). The cells are stirred on ice for 15 min and then centrifuged at 5000 rpms for 20 min.

*Purification of GST-tagged protein:* The centrifuged cell lysate is loaded onto a 2 mL glutathione-sepharose column (Pharmacia) and washed three times with 10 mL of 25 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DTT, 200 mM NaCl. The GST-tagged protein is eluted by 10 applications (1 mL each) of 25 mM Tris-HCl, pH 7.5, 10 mM reduced-glutathione, 100 mM NaCl, 1 mM DTT, 10% Glycerol and stored at -70°C.

*Kinase assay:* Tyrosine protein kinase assays with purified GST-c-Kit are carried out in a final volume of 30 µL containing 200-1800 ng of enzyme protein (depending on the specific activity), 20 mM Tris-HCl, pH 7.6, 3 mM MnCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, 1 mM DTT, 10 µM Na<sub>3</sub>VO<sub>4</sub>, 5 µg/mL poly(Glu,Tyr) 4:1, 1% DMSO, 1.0 µM ATP and 0.1 µCi [ $\gamma^{33}$  P] ATP. The activity is assayed in the presence or absence of inhibitors, by measuring the incorporation of  $^{33}$ P from [ $\gamma^{33}$  P] ATP into the poly(Glu,Tyr) substrate. The assay (30 µL) is carried out in 96-well plates at ambient temperature for 20 min under conditions described below and terminated by the addition of 20 µL of 125 mM EDTA. Subsequently, 40 µL of the reaction mixture is transferred onto Immobilon-PVDF membrane (Millipore, Bedford, MA, USA) previously soaked for 5 min with methanol, rinsed with water, then soaked for 5 min with 0.5% H<sub>3</sub>PO<sub>4</sub> and mounted on vacuum manifold with disconnected vacuum source. After spotting all samples, vacuum is connected and each well rinsed with 200 µL 0.5% H<sub>3</sub>PO<sub>4</sub>. Membranes are removed and washed 4 x on a shaker with 1.0% H<sub>3</sub>PO<sub>4</sub> and once with ethanol. Membranes are counted after drying at ambient temperature, mounting in Packard TopCount 96-well frame, and addition of 10

μL/well of Microscint TM (Packard). IC<sub>50</sub> values are calculated by linear regression analysis of the percentage inhibition of each compound in duplicate, at four concentrations (usually 0.01, 0.1, 1 and 10 μM). One  
5 unit of protein kinase activity is defined as 1 nmole of <sup>33</sup>P ATP transferred from [γ<sup>33</sup> P] ATP to the substrate protein per minute per mg of protein at 37°C.

The structure of the active agents identified by  
10 code nos., generic or trade names may be taken from the actual edition of the standard compendium "The Merck Index" or from databases, e.g., Patents International (e.g. IMS World Publications). The corresponding content thereof is hereby incorporated by reference.

15 Any reference hereinbefore and hereinafter to a free KIT tyrosine kinase inhibitor is to be understood as referring also to the corresponding pharmaceutically acceptable salts thereof, as appropriate and expedient.

The term "a gene whose expression level correlates  
20 with the biological activity of a KIT tyrosine kinase inhibitor" is understood to mean that the level of expression of such a gene in, e.g., a mammalian cell is related to the extent of inhibition of the KIT tyrosine kinase activity induced by an administered KIT tyrosine  
25 kinase inhibitor. In a more specific sense, this term is understood to mean that the level of expression of such a gene is related to the anti-tumor activity, especially anti-GIST activity, of an administered KIT tyrosine kinase inhibitor. A gene whose expression level  
30 correlates with the biological activity of a KIT tyrosine kinase inhibitor is preferably a gene listed in the Table below under Example 1, most preferably the Sprouty4A gene.



The term "biological sample" is meant to include any biological material separated from the mammalian body such as, e.g., tissue, cell lines, plasma, serum, and the like.

5        Preferably human is meant by the term "mammal" or "mammalian".

The term "ex vivo" means outside the body of the mammal.

The term "antibody that specifically binds to the  
10 Sprouty4A protein" refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds to the Sprouty4A protein. A molecule which specifically binds to the  
15 Sprouty4A protein is a molecule which binds to the Sprouty4A protein, but does not substantially bind to other molecules in a sample, e.g., a biological sample which naturally contains Sprouty4A. Preferably, antibodies that specifically bind to the Sprouty4A  
20 protein do also not bind to the Sprouty4C isoform, i.e., such antibodies are isoform-specific in that they only recognize epitopes which are present in Sprouty4A but not in Sprouty4C. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and  
25 F(ab')<sub>2</sub> fragments which can be generated by treating the antibody with an enzyme such as pepsin. The term "antibody" includes polyclonal and monoclonal antibodies, wherein the term "monoclonal antibody" refers to a population of antibody molecules that contain only one  
30 species of an antigen binding site capable of immunoreacting with a particular epitope. Scfv produced by genetic engineering are also encompassed by the term antibody.

According to another aspect of the present invention, the nucleic acid sequences identified as differentially regulated in tumor cells treated with  
5 imatinib can be employed to treat cancer (see Example 1, Tables 2-4). Specifically, in cases where imatinib gives rise to a beneficial therapeutic effect, nucleic acid molecules, or fragments thereof, containing genes which were upregulated after imatinib treatment may be  
10 administered to cancer cells to increase expression and thereby further inhibit tumor growth. Alterations in the physiological amount of the genes may act synergistically with chemotherapeutic agents used to treat cancer. In one embodiment, the nucleic acid molecules of the  
15 invention will be used to restore the gene expression to normal cellular levels or overexpress the gene in a population of malignant cells. In a preferred embodiment, the gene to be expressed is MAFbx.

In another embodiment, nucleic acid molecules may be  
20 used to decrease expression of genes found to be down-regulated after successful imatinib treatment (preferably SPRY4A), as yet another means to treat a malignancy (see Example 1, Tables 2-4). In this embodiment, oligonucleotides are targeted to specific regions of the  
25 genes that are critical for gene expression. The use of antisense oligonucleotides to decrease expression levels of a pre-determined gene is known in the art. In a particular embodiment, such antisense oligonucleotides  
are modified in various ways to increase their stability  
30 and membrane permeability, so as to maximize their effective delivery to target cells in vitro and in vivo. Such modifications include the preparation of

phosphorothioate or methylphosphonate derivatives, among many others, according to procedures known in the art.

In another embodiment, siRNAs directed to the genes may be employed to down regulate the expression of the genes. Selection of a suitable small interfering RNA (siRNA) molecule requires knowledge of the nucleotide sequence of the target mRNA, or gene from which the mRNA is transcribed. The siRNA molecules of the invention are typically between 12-30 nucleotides in length. More preferably, siRNA molecules are about 20, 21, 22, and 23 nucleotides in length. The siRNA molecules may comprise a sequence identical or at least 90% identical to any portion of the target gene whose expression is to be modulated including coding and non-coding sequences. A common method for identifying an siRNA target site within the target gene comprises scanning for AA dinucleotide sequences downstream of the AUG start codon and identifying the AA dinucleotide and the adjacent 3' 19 nucleotides as an siRNA target (see, for example, Ambion® Guidelines; Austin, TX; Ausubel et al., eds. Current Protocols in Molecular Biology, John Wiley and Sons, Inc., (1995)). Optionally, the identified sequence may be searched against a genome database such as GenBank® (maintained by The National Center for Biotechnology Information; NCBI) by a program such as BLAST® (Basic Local Alignment Search Tool) to ensure the sequence does not have significant homology to other genes.

The following examples present further detail regarding the practice of the instant invention. These examples are provided for illustrative purposes only and

are not intended to limit the scope of the invention in any way.

#### EXAMPLE 1

##### 5 Expression profiling of GIST cells treated with Imatinib using cDNA microarrays

###### Materials and Methods:

###### *GIST cell culture*

GIST882 cells were maintained in F-10 media  
10 supplemented with 15% FCS, 1% Bovine Pituitary Extract  
(Invitrogen, Carsbad, CA), 0.5% MITO+ Serum Extender  
(Becton Dickinson, Bedford, MA) and L-glutamine.

###### *GIST cells proliferation and apoptosis assessment*

15 For growth analysis, GIST882 cells were seeded at  
6.5 X 10<sup>5</sup> cells/60-mm dish. Imatinib (provided by  
Novartis Oncology and dissolved in water) was added  
directly to the media to achieve a final concentration of  
1 or 10 µM. Cells were refed with conditioned media  
20 containing imatinib or with conditioned media without  
drug every 12 h. Cells were harvested and stained for  
the cell number, cell viability, and induction of  
apoptosis using Guava ViaCount and Guava Nexin reagents  
(Guava Technology Inc., Burlingame, CA). The cells were  
25 counted using a Guava Personal Cytometer, and the data  
were analyzed using the Guava CytoSoft software package.

###### *Cell Cycle Analysis*

Cells were trypsinized, centrifuged, and fixed in  
30 70% ethanol at 4°C. Cell pellets were resuspended in 50  
µg/ml propidium iodide in PBS for 30 min at 4°C. The  
stained cells were analyzed by flow cytometry performed

on a FACScan, and the data were analyzed with Cell Quest software (Becton Dickinson).

#### *GIST cells treatment with Imatinib*

5        Imatinib mesylate was dissolved in water at a stock concentration of 10 mM. GIST882 cells were cultured to 60-70% confluency. Forty-eight hours prior to the treatment, cells were refed with complete media. Imatinib was added directly to the media to achieve the  
10 final concentration of either 1  $\mu$ M or 10  $\mu$ M.

#### *Total RNA preparation*

Imatinib treated and control GIST882 cells were harvested simultaneously at 60-80% confluency. Total RNA  
15 was isolated using guanidinium/isothiocyanate/phenol/chloroform method as previously described [Schultz et al., Cancer Res 56, 1997-2002. (1996)]. Total RNA was separated by agarose gel electrophoresis and visualized by ethidium bromide staining to check for integrity. 100  
20  $\mu$ g of total RNA was DNase treated using "DNA free" kit (Ambion, Austin, TX) according to manufacturer's specifications. Following DNase treatment the RNA was quantified and evaluated for integrity by agarose electrophoresis.

25

#### *Microarray preparation*

10,367 human cDNA fragments corresponding to known genes and ESTs from the I.M.A.G.E. consortium library (Research Genetics) were amplified by polymerase chain  
30 reaction (PCR), purified by isopropanol precipitation and resuspended in 50% DMSO at a concentration of 150 ng/ $\mu$ l. Arrays were spotted on GeneMachine Omnigrid arrayer (GeneMachine, San Carlos, CA) using poly-Lysine coated

glass slides. Slides were baked for 3 hours at 80°C in a vacuum oven, crosslinked in UV light (90 mJ) in Stratalinker (Stratagene, La Jolla, CA) and processed as described in Shalon et al., Genome Research 6 (7), 639-645, (1996) and DeRisi et al., Science 278 (5338), 680-686, (1997).

*Preparation and hybridization of the probe cDNA*

Fifteen micrograms of total RNA were reverse transcribed and amino allyl dUTP was incorporated in a reaction containing 500 ng oligo (dT) primers, 1x first strand buffer (Invitrogen, Carlsbad, CA), 0.01 M DTT, 500 µM each of dATP, dCTP, dGTP, and dTTP/aadUTP (2:3 ratio) 40 Units of rRNasin (Promega, Madison, WI) and 200 Units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). After brief denaturation and annealing of the primers at 70°C for 8 min, the reaction was incubated at 42°C for 2 hours, followed by alkali hydrolysis of RNA and cDNA purification using Microcon-30 columns (Millipore, Bedford, MA) according to the manufacturer's instructions. The cDNA was then labeled with either Cy3 or Cy5 dyes by a coupling reaction using FluoroLink™ Cy3 and Cy5 monofunctional dyes (Amersham Pharmacia Biotech, Piscataway, NJ) according to manufacture's specifications. Probes were purified using StrataPrep PCR Purification Kit (Stratagene, La Jolla, CA). Two of the samples (one labeled with Cy3 and one with Cy5) were combined, denatured, pre-annealed in the presence of 10 µg of Cot-1 DNA (Invitrogen, Carlsbad, CA) and 10 µg of poly-dA DNA, and hybridized to the cDNA microarrays overnight at 42°C in hybridization buffer (25% formamide, 5x SSC, 0.1% SDS and 100 µg/ml sonicated salmon sperm DNA). After hybridization, the arrays were washed 2

times in washing buffer A (2 x SSC, 0.1% SDS) at 42°C for 5 minutes and then 3 times in washing buffer B (0.1 x SSC, 0.1% SDS) at room temperature for 10 minutes. The slides were briefly dipped into distilled water and dried in a stream of nitrogen before scanning.

#### *Intensity extraction and data analysis*

Images were obtained by scanning the arrays in Affymetrix 428 scanners. Signal intensities for Cy3- and Cy5-labeled probes were extracted by ImaGene software package version 4.2 (BioDiscovery, Inc., Marina Del Rey, CA) using default settings and auto segmentation. Mean intensities for signal and background as well as quality characteristics ("empty" or "poor") of the spots were obtained at this time. The threshold for empty spots was achieved by raising threshold to a point when all blank spots were flagged. The formula for determining this value is as follows: if  $(M_S - M_B) / \sigma_B < \text{threshold}$ , then the spot is flagged, where  $M_S$  is the mean of signal,  $M_B$  is the mean of background and  $\sigma_B$  is the Standard Deviation of the background. The "poor" spots were calculated using the following formula: if  $\sigma_S / M_S > \text{threshold}$ , then the spot is flagged ( $\sigma_S$  is the Standard Deviation of the signal and  $M_S$  is the Mean of signal). The threshold was set at 0.4 to determine "poor" spots. The data were then analyzed using GeneSight software package version 3.0.4 (BioDiscovery, Inc., Marina Del Rey, CA). Data preparation consisted of the following steps: 1) background correction was performed by subtracting the local group median (clique size = 5) background from the signal of each spot; 2) spots that were flagged as "empty" and "poor" were omitted from the analysis; 3) the data were normalized using Piece-wise linear

normalization, which was done using 10 bins and a minimum of 100 elements in each bin; 4) the intensity ratio of two channels was calculated; 5) the  $\log_2$  was calculated for each ratio; and 6) the two channels were normalized by subtracting the  $\log_2$  of the mean of the intensity of the total signal from each individual spot intensity. At least two "flip-dye" experiments were performed for each time point and the mean of the repeated experiments were used in the final data analysis. Finally, the mean ratio for each spot was calculated for all time points and the standard deviation and coefficient of variance were calculated for these values.

*cdna microarray clone verification and sequence analysis*

cdna fragments of interest were obtained from original stock plates used for microarray fabrication by either plasmid isolation or direct amplification of the fragments from the bacteria. Clones were re-sequenced and the correct annotation and homology was identified using the basic local alignment search tool (BLAST, NCBI) against GenBank/EMBL database. DNA and amino acid sequence analyses were performed with the Wisconsin Package version 9.1 (Genetics Computer Group).

#### *Northern Blotting*

Fifteen micrograms of total RNA was used for standard Northern blot analysis, as previously described [Schultz et al., Cancer Res 56, 1997-2002. (1996)]. Blots were hybridized with a 1,454 bp  $^{32}\text{P}$ -labeled SPRY4A cdna probe, corresponding to nucleotides 2594-4048 of GenBank entry AF227516.



### Reverse transcription of RNA

Two micrograms of DNase treated RNA were reverse transcribed using M-MuLV reverse transcriptase (NEB, Beverly, MA) in a 20  $\mu$ l reaction containing 1 x RT Buffer (NEB, Beverly, MA), 0.5 mM each dNTPs, 4  $\mu$ M oligo-dT (16-18) primer, 10 units RNase inhibitor (Promega, Madison, WI) and 200 units of reverse transcriptase. Primers were pre-annealed for 10 min at 70°C, and the reaction incubated for 1 hr at 42°C followed by enzyme inactivation for 10 min at 90°C.

### Polymerase chain reaction

DNA fragments of SPRY4A/C, SPRY4A and  $\beta$ -actin were amplified by polymerase chain reaction (PCR) using cDNAs obtained by reverse transcription of mRNA from GIST cells and tumor biopsies as a template and the following sets of primers: SPRY4A - forward 5'-CCGTTCTGTGGAGAGTCGATTAC-3' (SEQ ID NO: 1), reverse 5'-GTCCCTCAGTGGCTCTCGACT-3' (SEQ ID NO: 2); Sprouty4A/4C (isoform determining) - forward 5'-ACCATCCTACCCATTGACCA-3' (SEQ ID NO: 3), reverse 5'-GGCTTCGACACAACTGTCA-3' (SEQ ID NO: 4);  $\beta$ -actin - forward 5'-CTCACCATGGATGATGATATCGC-3' (SEQ ID NO: 5), reverse 5'-CATGATGGAGTTGAAGGTAGTTTCGT-3' (SEQ ID NO: 6). PCR was performed in a reaction volume of 30  $\mu$ l containing cDNA from 1  $\mu$ l of the reverse transcription reaction described above as a template, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin, 0.5  $\mu$ M of both the forward and reverse primer, 60  $\mu$ M of each deoxyribonucleotide, 5% dimethyl sulfoxide, and 0.5U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Following an initial denaturation step at 95°C for 5 min, DNA was amplified through 21, 23, 25, and 27 cycles consisting of 5 sec denaturing at 95°C, 60 sec annealing

at 55°C, and 90 sec extension at 72°C. The products were resolved on a 1.5% agarose gel and were visualized by UV light following staining with Ethidium Bromide. Images of the gels were obtained using Alpha Imager 2200  
5 documentation and analysis system (Alpha Innotech, San Leandro, CA). Images were captured within linear dynamic range and controlled for white color saturation. DNA bands were quantified using Alpha Imager v5.5 software package and Fuji Image Gauge v3.11 software package (Fuji  
10 Photo Film Co., Ltd).

#### Results:

The response of GIST882 cells to imatinib was assessed *in vitro*. Both 1 and 10  $\mu$ M imatinib  
15 dramatically inhibited cell growth within 24-48 h after exposure (Fig. 2A). However, GIST cells treated with imatinib show no significant increase (<3% of total cell number) in either early or late apoptosis during 96-  
h treatment period as determined by annexin-V and 7  
20 amino-actinomycin D staining. The fraction of viable cells was  $93 \pm 5\%$  for both treated and untreated cells during entire course of treatment (data not shown). A cell cycle analysis of drug-treated cells was also  
performed and a 57% (15.5% versus 6.6% of total cell  
25 amount) decrease in the amount of cells in S phase with addition of 1 or 10  $\mu$ M of imatinib was found (Table 1). The variation of the total number of cells in S phase was  
<5% for independently repeated experiments.

Table 1

	<u>72 hours</u>			<u>96 hours</u>		
	G0-G1	G2-M	S	G0-G1	G2-M	S
Untreated	74.72%	9.28%	15.99%	74.33%	10.63%	15.04%
5 1 $\mu$ M imatinib	84.21%	8.53%	7.25%	79.29%	13.48%	7.22%
10 $\mu$ M imatinib	85.70%	7.87%	6.43%	80.87%	12.23%	6.90%

Table 1: Percentage of GIST882 cells in indicated phase of cell cycle at the 72 and 96 hour time points.

10

To identify potential imatinib-specific genetic targets, a human GIST cell line (GIST882) was treated with 10  $\mu$ M imatinib for 0, 6, 24, or 48 hr. RNA samples labeled with Cy3- or Cy5-dyes were hybridized to 10,368-  
 15 element cDNA microarrays containing known genes and ESTs. 148 genes or ESTs (expressed sequence tags) were found to be differentially regulated in at least one timepoint with 95% confidence (Table 2). 62 genes displayed significant down-regulation at all time points tested  
 20 (Table 3). 14 genes were identified, which displayed a significant change (at least 2.5-fold) in expression levels at all time points following treatment (Table 4). Two genes were upregulated and 12 genes were down-regulated (see Table 4). The mean intensity ratio was  
 25 calculated for all spots during the course of drug treatment and it was found that out of the 12 genes which showed decreased expression following drug treatment, only one transcript was dramatically down-regulated at each time point (from 4- to 13-fold) (Fig. 1).  
 30 Therefore, the studies were focused on this gene.

Table 2

Upregulated Genes

- AA009593--ESTs
- 5 AA011673--ESTs, Weakly similar to ALU8\_HUMAN ALU SUBFAMILY SX SEQUENCE  
CONTAMINATION WARNING ENTRY [H.sapiens]  
AA057425--myeloid/lymphoid or mixed-lineage leukemia (trithorax (Drosophila) homolog);  
translocated to, 2  
AA100674 | AI732414--ESTs
- 10 AA148532--Homo sapiens, Similar to CG7083 gene product, clone MGC:10534, mRNA, complete cds  
AA416785--heterogeneous nuclear ribonucleoprotein A1  
AA423978--ESTs, Weakly similar to JM27 [H.sapiens]  
AA427528--zinc-finger protein ZBRK1  
AA454597--golgi membrane protein GP73
- 15 AA455497--protein tyrosine phosphatase, receptor type, C  
AA457700--stearoyl-CoA desaturase (delta-9-desaturase)  
AA463256--ESTs  
H09124--Homo sapiens cDNA: FLJ23573 fis, clone LNG12520  
H09164--Homo sapiens cDNA: FLJ22030 fis, clone HEP08669
- 20 H25042--chromosome 8 open reading frame 1  
N21407--activated RNA polymerase II transcription cofactor 4  
N23606--SRX (sex determining region Y)-box 4  
N30157--ESTs  
N50935--hypothetical protein ASH1
- 25 N69100--EST  
R97944--zinc finger protein 26 (KOX 20)  
T41066--ESTs  
W69669--chromosome 21 open reading frame 4

30 Downregulated Genes

- AA004595--ESTs  
AA011041--ESTs  
AA022558--Homo sapiens cDNA: FLJ22120 fis, clone HEP18874
- 35 AA022949--fibroblast growth factor 18  
AA043878--KIAA1151 protein  
AA055491--transmembrane 4 superfamily member (tetraspan NET-2)  
AA055971--KIAA0810 protein  
AA083207--EST
- 40 AA101833--ESTs  
AA128617--hypothetical protein FLJ20898  
AA131530--ESTs  
AA133215--receptor (calcitonin) activity modifying protein 1  
AA133778--ESTs
- 45 AA149637--transducin (beta)-like 1  
AA158375--SRP25 nuclear protein  
AA164229 | AI732625--ESTs  
AA181898--hypothetical protein FLJ20186  
AA206050--ESTs
- 50 AA283599--CGI-94 protein  
AA286905--checkpoint with forkhead and ring finger domains  
AA287828--up-regulated by BCG-CWS  
AA398262--ESTs, Weakly similar to AAB47496 NG5 [H.sapiens]  
AA401470--hypothetical protein RP4-622L5
- 55 AA412477--EST  
AA417011--EST  
AA418418--hypothetical protein FLJ10055  
AA425382--sprouty (Drosophila) homolog 4

- AA427395--Homo sapiens dpy-30-like protein mRNA, complete cds  
 AA427767 | AI732744--RNA binding motif protein 14  
 AA427778--complement C1r-like proteinase precursor,  
 AA432064--ESTs
- 5 AA446893--Homo sapiens cDNA FLJ10705 fis, clone NT2RP3000850  
 AA446906--solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34kD),  
 member 17  
 AA447746--hypothetical protein  
 AA453287--hypothetical protein MGC5306
- 10 AA453616--Homo sapiens AKAP-associated sperm protein (ASP) mRNA, complete cds  
 AA453994--NPD007 protein  
 AA454149--EST  
 AA455042--chemokine-like factor, alternatively spliced  
 AA455275--serologically defined breast cancer antigen NY-BR-87
- 15 AA455291--ESTs  
 AA457138--ESTs  
 AA463444--RB-associated KRAB repressor  
 AA463516--lysophosphatidic acid acyltransferase-delta  
 AA464578--Homo sapiens, clone MGC:3182, mRNA, complete cds
- 20 AA464736--Homo sapiens, clone MGC:10710, mRNA, complete cds  
 AA478630--Huntingtin interacting protein C  
 AA479155--ESTs  
 AA485357--ESTs  
 AA486445--hypothetical protein FLJ10120
- 25 AA487465--  
 AA487527--KIAA0937 protein  
 AA488650--TRAF and TNF receptor-associated protein  
 AA598625--solute carrier family 1 (glutamate/neutral amino acid transporter), member 4  
 AA599043--ESTs
- 30 AA609422--EST  
 AA620488--  
 AA625765--hypothetical protein MGC2594  
 AA757671--  
 AA778683--ESTs
- 35 AA876219--phosphodiesterase 2A, cGMP-stimulated  
 AA932870--zinc finger protein 143 (clone pHZ-1)  
 AI015536--mitochondrial ribosomal protein L12  
 AI075273--phosphodiesterase 7A  
 AI094859--engrailed homolog 2
- 40 AI129421--  
 AI218900--H4 histone family, member I  
 AI337297--tumor suppressing subtransferable candidate 3  
 H01164--serine/threonine kinase 17a (apoptosis-inducing)  
 H09082--ESTs
- 45 H09241--ESTs  
 H11051--copine VI (neuronal)  
 H11918--KIAA0534 protein  
 H16789--CGI-152 protein  
 H18934--Human DNA sequence from clone 321D2 on chromosome 16. Contains a gene for a
- 50 Ribosomal Large Subunit Pseudouridine Synthase (EC 4.2.1.70, Pseudouridylate Synthase,  
 Uracil Hydrolase) LIKE protein, a gene for a novel protein similar to replication facto
- H28794--ESTs  
 H49519--ESTs  
 H52062--hypothetical protein FLJ22313
- 55 H63534--methylmalonate-semialdehyde dehydrogenase  
 H84926--  
 H92504--hypothetical protein  
 N21081--HMBA-inducible  
 N22836--ESTs

- N29696--EST  
 N34470--ESTs  
 N38992--GATA-binding protein 4  
 N39077--ESTs  
 5 N39573--KIAA1183 protein  
 N48070--ESTs  
 N51651--KIAA0909 protein  
 N56906--EST  
 N62376--ESTs  
 10 N63375--EST  
 N68864--ESTs, Weakly similar to ALU8\_HUMAN ALU SUBFAMILY SX SEQUENCE  
 CONTAMINATION WARNING ENTRY [H.sapiens]"  
 N70837--KIAA1275 protein  
 N73571--  
 15 N81032 | AJ822124--  
 N93057 | AI668684--ESTs  
 R16053--HMG-box containing protein 1  
 R19306--nuclear factor I/X (CCAAT-binding transcription factor)  
 R34224--ESTs  
 20 R43308--Homo sapiens mRNA; cDNA DKFZp434D0720 (from clone DKFZp434D0720)  
 R44562--KIAA0564 protein  
 R78530 | R78490--Homo sapiens cDNA: FLJ22380 fis, clone HRC07453, highly similar to  
 HUMCLPB Homo sapiens CLP mRNA  
 R85261--ESTs  
 25 R86847--FH1/FH2 domain-containing protein  
 T49802--hypothetical protein MGC2477  
 T55569--hypothetical protein FLJ11773  
 T60082--CpG binding protein  
 T82453--ESTs, Weakly similar to T12482 hypothetical protein DKFZp564P0662.1 [H.sapiens]  
 30 T83646--phosphoglycerate dehydrogenase  
 T94848 | AJ732248--ESTs  
 T96985--hypothetical protein MGC4692  
 W19822--semaphorin Y  
 W37689--ESTs, Weakly similar to weak similarity to TPR domains [C.elegans]  
 35 W48838--hypothetical protein MGC4175  
 W56308--EST  
 W67228--ESTs  
 W84774--ESTs  
 W87801--ESTs  
 40 W87939--ESTs  
 W89128--ESTs  
 W93024--Homo sapiens clone IMAGE:112574 mRNA sequence  
 W93943--ESTs  
 W94591--ESTs  
 45

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Table 2: GenBank Accession numbers and brief descriptions, where available

Table 3

	AA039851--protein tyrosine phosphatase type IVA, member 3
	AA417373--2,4-dienoyl CoA reductase 2, peroxisomal
	AA446906--solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34kD),
5	member 17
	AA464578--Homo sapiens, clone MGC:3182, mRNA, complete cds
	AA598625--solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
	AA776942--coatamer protein complex, subunit epsilon
	AA876219--phosphodiesterase 2A, cGMP-stimulated
10	AI190798--keratin, hair, acidic,2
	AI218900--H4 histone family, member I
	H98755--ESTs, Weakly similar to unknown protein [R.norvegicus]
	N62400--heat shock 90kD protein 1, alpha
	N62979--ESTs, Highly similar to T00391 hypothetical protein KIAA0612 [H.sapiens]
15	N71692--ESTs, Weakly similar to ALUC_HUMAN [H.sapiens]"
	R78530   R78490--Homo sapiens cDNA: FLJ22380 fis, clone HRC07453, highly similar to
	HUMCLPB Homo sapiens CLP mRNA
	T82453--ESTs, Weakly similar to T12482 hypothetical protein DKFZp564P0662.1 [H.sapiens]
	W57983--pinin, desmosome associated protein
20	AA004595--ESTs
	AA043878--KIAA1151 protein
	AA128617--hypothetical protein FLJ20898
	AA133215--receptor (calcitonin) activity modifying protein 1
	AA158375--SRp25 nuclear protein
25	AA164229   AI732625--ESTs
	AA255876--zinc finger protein ANC_2H01
	AA293215--ubiquinol-cytochrome c reductase core protein I
	AA398332--EST
	AA401341--chromosome 16 open reading frame 5
30	AA412477--EST
	AA417011--EST
	AA425382--sprouty (Drosophila) homolog 4
	AA427570--NADH dehydrogenase (ubiquinone) flavoprotein 1 (51kD)
	AA447746--hypothetical protein
35	AA453994--NPD007 protein
	AA455523--
	AA457138--ESTs
	AA460274--DKFZP434P106 protein
	AA485357--ESTs
40	AA487301--ESTs
	AA487465--
	AA490249--arginine-glutamic acid dipeptide (RE) repeats
	AA598781--matrix Gla protein
	AA609422--EST
45	AI015536--mitochondrial ribosomal protein L12
	AI125840--leucine-rich neuronal protein
	H48165--ESTs
	H49519--ESTs
	H63534--methylmalonate-semialdehyde dehydrogenase
50	N21385--choroideremia-like (Rab escort protein 2)
	N34470--ESTs
	N51651--KIAA0909 protein
	N62908--ESTs
	N63375--EST
55	N67305--ESTs
	N70837--KIAA1275 protein
	N81032   AJ822124--
	R19306--nuclear factor I/X (CCAAT-binding transcription factor)

- R44840--ESTs  
 T55569--hypothetical protein FLJ11773  
 T64323--  
 T83646--phosphoglycerate dehydrogenase  
 5 W32281--CGI-39 protein; cell death-regulatory protein GRIM19  
 W74725--Homo sapiens mRNA; cDNA DKFZp434A1319 (from clone  
 DKFZp434A1319); complete cds  
 W87939--ESTs

- 10 Table 3: GenBank Accession numbers and brief descriptions, where available

Table 4

ACC	Clone ID	6 hours	12 hours	24 hours	48 hours	Mean ratio	CV
AF227516	Sprouty (Drosophila) homolog 4	-3.31	-1.91	-3.73	-2.38	-2.83 ± 0.83	0.29
NM_031866	Frizzled (Drosophila) homolog 8	-2.22	-1.39	-2.54	-0.97	-1.78 ± 0.72	0.41
AF335324	RTP801	-2.11	-1.17	-1.67	-1.35	-1.58 ± 0.41	0.26
AC106791	EST chromosome 5	-1.89	-0.67	-2.29	-1.46	-1.58 ± 0.69	0.44
NM_024600	Hypothetical protein FLJ20898	-1.63	-0.45	-2.5	-1.49	-1.52 ± 0.84	0.55
AC124312	EST (chromosome15)	-0.93	-1.43	-1.72	-1.7	-1.45 ± 0.37	0.26
BC020567	GEF (Rho/Rac guanine exchange factor)	-1.95	-0.8	-1.39	-1.49	-1.41 ± 0.48	0.34
T82453	EST	-0.86	-1.03	-2.42	-0.99	-1.32 ± 0.73	0.55
AA487465	EST	-0.87	-0.81	-1.53	-1.99	-1.30 ± 0.57	0.44
AA598625	Solute carrier family 1	-0.34	-0.88	-2.93	-1.01	-1.29 ± 1.13	0.88
NM_002599	Phosphodiesterase 2A, cGMP-stimulated	-0.45	-0.72	-2.63	-1.25	-1.26 ± 0.97	0.77
N49836	EST	-1.57	-0.96	-0.96	-1.51	-1.25 ± 0.34	0.27
AY059629	MAFbx	1.81	1.13	1.61	0.72	1.32 ± 0.49	0.37
H70163	EST	0.92	2.16	1.93	0.49	1.38 ± 0.80	0.58

- Table 4: cDNA microarray analysis of imatinib treated  
 15 GIST 882 cells. Shown are the log<sub>2</sub> intensity ratios for each time point and the overall mean log<sub>2</sub> ratio for each gene. StDev = Standard deviation, CV = Coefficient of variance, EST = Expressed sequence tag, ACC = GenBank Accession Number.

20

- Upon sequencing and database evaluation, it was found that the sequence of the spotted cDNA fragment matched two isoforms of Sprouty4, 4A and 4C (Figs. 2B). SPRY4A (GenBank Accession No. AF227516) and SPRY4C  
 25 (GenBank Accession No. AF227517) represent alternatively spliced forms of the same gene. The SPRY4A transcript encodes for a 322 amino acid protein, while SPRY4C



transcript encodes for a 106 amino acid polypeptide (Fig. 2B). The SPRY4A transcript includes all of the SPRY4C sequence with extended amino and carboxy termini.

In support of the microarray results, a cDNA probe  
5 was derived that would detect both the ~5,000 base (SPRY4A) and the ~7,000 base (SPRY4C) transcripts of Sprouty4. Northern blot analysis indicated that SPRY4A was down-regulated following treatment with 10  $\mu$ M imatinib (Fig. 2C). In comparison, SPRY4C transcript was  
10 not detected by Northern blotting (data not shown). A set of oligonucleotide PCR primers were designed that can differentiate between the two Sprouty4 transcripts (Fig. 2B). The oligonucleotide primer pair 1F/1R is predicted to yield a RT-PCR product of ~500 bp that would represent  
15 both SPRY4A and 4C. In comparison, primer pair 2F/2R would yield a fragment of 812 bp for SPRY4A and a 358 bp fragment for SPRY4C (Fig. 2B). The RT-PCR analysis confirmed that SPRY4A, and not 4C was constitutively expressed in GIST cells and exhibited dramatic down-  
20 regulation after drug treatment (Fig. 2D).

## Example 2

Imatinib inhibits SPRY4A expression through down-regulation of c-KIT, ERK1/2 and AKT

### 25 Materials and Methods:

*Cell lysate preparation, SDS-PAGE and Western blot analysis*

Anti- $\beta$ -actin was purchased from Sigma (St. Louis, MO) and was probed in 1:5000 dilution in 5% dried milk.  
30 Anti-phospho-c-Kit (Tyr719) was purchased from Cell Signaling (Beverly, MA) and was probed in 1:500 dilution in 5% BSA. Anti-c-KIT was purchased from Santa Cruz (Santa Cruz, CA) and was used in 1:100 dilution in 5%

dried milk. Anti-phospho-ERK1/2 was purchased from Cell Signaling (Beverly, MA), anti-ERK1, and anti-ERK2 were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA), anti-AKT and anti-AKT/phosphoThr308 antibodies were  
5 obtained from Cell Signaling Technology (Beverly, MA) and were probed in 1:1000 dilution in 5% BSA.

Cells at 60-70% confluence were washed twice with ice-cold D-PBS before scraping on ice with Lysis Buffer [20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 2.5 mM Na-  
10 pyrophosphate; 1 mM Na- $\beta$ -glycerophosphate; 5 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 1% Triton X-100, and 1 tablet of protease inhibitor cocktail (Roche, Indianapolis, IN) per 40 ml lysis buffer]. Cellular debris was removed by centrifugation (14,000 x g for 15 min at 4°C), and  
15 quantitation of protein was performed using a bicinchoninic acid/copper (II) sulfate assay (Sigma, St. Louis, MO). Fifty micrograms of total protein from cell extracts and tissues were separated by standard SDS-PAGE and transferred to Immobilon-P (polyvinylidene  
20 difluoride; Millipore, Bedford, MA). Prior to antibody probing, the membranes were blocked in 5% dried milk. The primary antibodies were diluted 1:1000 (unless other specified), and the HRP-conjugated secondary antibodies were diluted 1:10,000 (Amersham, Piscataway, NJ). NEN  
25 Renaissance Enhanced Luminol Reagents (Boston, MA) were used as substrates for detection. For reuse of the same membrane with another primary antibody, the membrane with stripped with Restore Western Blot Stripping buffer (Pierce, Rockford, IL).

30

#### Results:

GIST882 cells were examined for activation of c-KIT, ERK1/2 and AKT using anti-phosphorylated antibodies. c-

KIT, ERK1/2 and AKT were found to be constitutively activated in exponentially growing GIST882 cells (Fig. 3A). The inhibitory efficacy of the drug on the activity of c-KIT, ERK1/2 and AKT was then evaluated with 5 different drug concentrations (1 and 10  $\mu$ M of imatinib) at various time intervals. It was found that either drug concentration resulted in the loss of phosphorylated c-KIT within 30 minutes without affecting the total level of KIT protein (Fig. 3A). Imatinib also potently 10 inhibited the constitutive activation of ERK1/2 (ppERK1/2) without affecting total cellular levels of ERK1/2 (Fig. 3A). Inhibition of AKT took place within 30 minutes for 10  $\mu$ M of imatinib and 1 hour for 1  $\mu$ M without affecting the total level of AKT (Fig. 3A). Imatinib 15 effects on SPRY4A mRNA expression in GIST882 were evaluated next. At both concentrations of drug, SPRY4A levels were noticeably decreased by 3 hrs and virtually undetectable by 6 hrs (Fig. 3B). Therefore, it was shown in GIST882 cells that imatinib treatment resulted in 20 decreased autophosphorylation of the mutant c-KIT polypeptide by inhibiting c-KIT kinase activity rather than by down-regulating expression of the c-KIT protein. Furthermore, this inhibition leads to down-regulation of the activation of ERK1/2 and AKT and decreased levels of 25 SPRY4A mRNA.

### Example 3

SPRY4A expression is regulated by the ERK1/2 signaling pathway independent of AKT

#### 30 Materials and Methods:

*GIST cells treatments with MEK inhibitor*

The MEK1/2 inhibitor U0126 was purchased from Promega (Madison, WI) and dissolved in DMSO at a stock

concentration of 15 mM. GIST cells were cultured to 60-70% confluency. Forty-eight hours prior to the treatment, the media was replaced. MEK inhibitor was added directly to the media to achieve the final  
5 concentration of 1  $\mu$ M, 10  $\mu$ M and 30  $\mu$ M.

### Results:

Previous studies have shown that the ERK pathway positively regulates the expression of the Sprouty genes  
10 in mouse cells, and that in a limited number of tumor cell lines which exhibit constitutive activation of ERKs, SPRY1 and/or SPRY2 mRNA is elevated [Ozaki et al., Biochem Biophys Res Commun 285, 1084-8. (2001)]. To determine if ERK1/2 pathways also regulated SPRY4A in  
15 tumor cells, the GIST882 line was treated with varying concentrations of U0126, a MEK inhibitor, for 6 hrs and 24 hrs and the expression of SPRY4A was evaluated. It was found that SPRY4A levels were decreased ~90% and ~70% by 6 and 24 hrs, respectively, at a concentration of 30  
20  $\mu$ M U0126 (data not shown). Lower doses of U0126 had minimal effect on SPRY4A levels at either of the time points (data not shown). The levels of constitutively activated ERK1/2 following treatment with 30  $\mu$ M U0126 for 30 min, 1, 3, and 6 hrs were evaluated next. This  
25 treatment resulted in a complete suppression of activated ERKs by 30 min; expression of SPRY4A was reduced by 3 hrs and was nearly absent by 6 hrs (Fig. 4A and 4B). The level of SPRY4A down-regulation was comparable to that seen with imatinib, except that the duration of  
30 suppression with a single dose of imatinib was more prolonged (data not shown). Interestingly, U0126 inhibits phosphorylation of AKT shortly after treatment but this effect was transient and the amount of phospho-

AKT returned to the initial level within 3 hours of treatment (Fig. 4A). Furthermore, the presence of U0126 did not affect the levels of total AKT. These results further confirm that constitutive activation of ERK1/2 through the c-KIT pathway leads to expression of SPRY4A and that this process may be independent of AKT signaling.

#### Example 4

##### 10      **SPRY4A and MAFbx expression in clinical GIST samples**

###### Materials and Methods:

###### *Clinical samples*

Tumor specimens were obtained from seven patients enrolled in the CSTI571-B2222 clinical trial sponsored by  
15      Novartis Oncology. Eligibility criteria included histological confirmation of GIST with documentation of c-KIT expression, as well as evidence of unresectable recurrent and/or metastatic disease. After signing informed consent, patients were evaluated to confirm  
20      acceptable hematologic, renal and hepatic function. Patients were then randomized to receive 400 mg or 600 mg per oral once daily dosing of imatinib. Ultrasound-guided 14 gauge core biopsies of non-necrotic tumor were obtained prior to therapy initiation and while on drug  
25      for between 1-28 days. Specimens were flash frozen and kept in liquid N<sub>2</sub>. Patients were monitored for response to imatinib mesylate at 4-12 week intervals.

Patients #1-3 had GIST of either the stomach or the small bowel and had a partial response to imatinib  
30      treatment defined as greater than or equal to 50% decrease from baseline in the sum of products of perpendicular diameters of all measurable lesions. Patient #4 had a partial response to imatinib initially

but developed disease progression 3 months after initiation of the treatment. The patient was taken off drug and a core tumor biopsy was obtained. Patients #5 and #6 presented with large intestine and gastric GIST, respectively, and they had no response to imatinib treatment. Patient #5 remained with stable disease and Patient #6 developed disease progression. Additional tumor was obtained from patient #6 during palliative surgical resection approximately one week after discontinuing drug therapy. Patient #7 had two measurable abdominal tumors, one of which responded to therapy (R) where as the other progressed (NR).

Total RNA from the tumor specimens was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) using manufacturer's protocol. See other examples for RT-PCR conditions. Protein extracts from a patient with a myxoid extremity liposarcoma and GIST patient #6 tumor were prepared as described above.

## 20 Results:

Core biopsy tumor specimens taken from imatinib-treated GIST patients (as described in Methods) were evaluated for expression of SPRY4A by RT-PCR analysis. The expression levels of SPRY4 were dramatically decreased following imatinib therapy in tumors from patients (#1, 2, 3, and 7(R)) who showed a favorable clinical response to the drug (Fig. 5A). In comparison, the imatinib-resistant patients (#5, 6, and 7 (NR)) expressed similarly high levels of SPRY4A both pre- and post-treatment. In the patient (#4) who initially responded to the drug treatment but subsequently relapsed, the SPRY4A levels in the tumor decreased dramatically for the duration of the favorable clinical

response but returned to the pre-treatment levels during disease progression (Fig. 5A).

The expression level of MAFbx in clinical samples was also assessed (patients 1, 4, 6, and 7). In patients 5 1 and 7(R), both of whom showed favorable clinical responses, high levels of MAFbx transcripts were detected in the treated tumor. Notably, the NR tumor from patient 7 showed low/minimal levels of MAFbx. In the tumor sample from patient 4, the level of MAFbx increased 10 during the response but returned to pretreatment level during the disease progression. In patient 6, who never responded to the drug, the level of MAFbx was not changed (i.e., not detected) in the paired samples (Fig. 5B).

In patient #6, sufficient tissue from the non- 15 responding tumor was available to evaluate the c-KIT, AKT and ERK1/2 protein levels. A western blot of the drug resistant GIST indicated a continued expression of activated forms of c-KIT, AKT and ERK1/2 during imatinib therapy (Fig. 6A, B, and C). In comparison, a control 20 specimen taken from an untreated patient with the histological diagnosis of myxoid liposarcoma, a sarcoma histotype that is clinically unresponsive to imatinib, failed to express detectable levels of c-KIT (Fig. 6A). Importantly, in this control liposarcoma tissue, ERK1/2 25 was constitutively activated (Fig. 6B) and SPRY4A was expressed at a level comparable to a typical GIST specimen (data not shown). These results suggest that constitutive activation of ERK1/2 in GISTs and in a liposarcoma, whether c-KIT-dependent or -independent, 30 contribute to expression of SPRY4A. It was determined that the tumor from patient #6 possessed an in frame mutation (1530ins6) in exon 9 of c-KIT. Together, these results suggest that SPRY4A is regulated in GISTs by the

c-KIT and ERK1/2 pathways and can be used as a genetic marker of clinical response to imatinib treatment for these tumors. This exon 9 insertion of c-KIT may be associated with GIST cells to respond which are  
5 refractory to treatment.

### Example 5

#### Imatinib responses of other genes

##### Materials and Methods:

##### 10 Reverse Transcription of RNA

Two  $\mu$ g of DNase treated RNA were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Beverly, MA) in a 20- $\mu$ l reaction containing 1X reverse transcription buffer (NEB), 0.5 mM  
15 each dNTP, 4  $\mu$ M oligo(dT)<sub>16-18</sub> primer, 10 units of RNase inhibitor (Promega), and 200 units of reverse transcriptase. Primers were preannealed for 10 min at 70°C, and the reaction was incubated for 1 h at 42°C, followed by enzyme inactivation for 10 min at 90°C.

20

##### PCR

DNA fragments of SPRY4A, MAFbx, FZD8, PDE2A, RTP801, FLJ20898, ARHGEF2, and  $\beta$ -actin were amplified by PCR using cDNAs obtained by reverse transcription of  
25 mRNA from GIST cells and tumor biopsies as a template and the following sets of primers: Sprouty 4A (SPRY4A), 5'-CCGTTCTGTGGAGAGTCGATTTAC-3' (SEQ ID NO: 1) and 5'-GTCCCTCAGTGGCTCTCGACT-3' (SEQ ID NO: 2); Frizzled 8 (FZD8), 5'-ACAGTGTGATTGCTATTAGCATG-3' (SEQ ID NO: 7) and  
30 5'-GTGAAATCTGTGTATCTGACTGC-3' (SEQ ID NO: 8); ARHGEF2, 5'-AAGGACGGAGAAAGGGAGAA-3' (SEQ ID NO: 9) and 5'-CAAGACAGCAGTGACCCTGA-3' (SEQ ID NO: 10); PDE2A, 5'-CCGCGATCTTTCTCGTAGTC-3' (SEQ ID NO: 11) and 5'-



CCCACTTCTGCTACCTGCTC-3' (SEQ ID NO: 12); MAFbx, 5'-  
 GTCCTGGGGTGAAAGTGAAA-3' (SEQ ID NO: 13) and 5'-  
 TCACAGCTCACATCCCTGAG-3' (SEQ ID NO: 14); FLJ20898, 5'-  
 CCCGAGTGACTCTGTTTTCC-3' (SEQ ID NO: 15) and 5'-  
 5 ACACCCAGTTGGAGGTGAAG-3' (SEQ ID NO: 16); RTP801, 5'-  
 AGACACGGCTTACCTGGATG-3' (SEQ ID NO: 17) and 5'-  
 TTGATGACTCGGAAGCCAGT-3' (SEQ ID NO: 18); and  $\beta$ -actin, 5'-  
 CTCACCATGGATGATGATATCGC-3' (SEQ ID NO: 19) and 5'-  
 CATGATGGAGTTGAAGGTAGTTTCGT-3' (SEQ ID NO: 20). PCR was  
 10 performed in a reaction volume of 30  $\mu$ l containing cDNA  
 from 1  $\mu$ l of the reverse transcription reaction described  
 above as a template, 10 mM Tris-HCl (pH 8.3), 50 mM KCl,  
 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin, 0.5  $\mu$ M of both the forward  
 and reverse primer, 60  $\mu$ M of each deoxyribonucleotide,  
 15 5% DMSO, and 0.5 unit of platinum Taq DNA polymerase  
 (Invitrogen). After an initial denaturation step at 95°C  
 for 5 min, DNA was amplified through 19, 21, 22, 23, 24,  
 25, and 27 cycles consisting of 5-s denaturing at 95°C,  
 60-s annealing at 55°C, and 90-s extension at 72°C. The  
 20 products were resolved on a 1.5% agarose gel and  
 visualized by UV light after staining with ethidium  
 bromide. Images of the gels were obtained using an Alpha  
 Imager 2200 documentation and analysis system (Alpha  
 Innotech, San Leandro, CA). Images were captured within  
 25 linear dynamic range and controlled for white color  
 saturation. DNA bands were quantified using the Alpha  
 Imager version 5.5 software package and the Fuji Image  
 Gauge version 3.11 software package (Fuji Photo Film Co.,  
 Ltd.).

30

#### Results:

To further elucidate the role of the members of the SPRTY family, RT-PCR was performed on SPRY1, 2, 3, and 4

in untreated and treated imatinib GIST cells. As seen in Figure 7A, SPRY1, 2, and 3 mRNA levels are relatively low and increased following treatment with imatinib. This is in contrast to the loss of expression of SPRY4.

5 Additionally, a Western blot was performed on lysates from GIST cells that had been treated with imatinib for 24, 72, and 120 hours (Fig. 7B). Notably, the protein is nearly absent by 24 hours and is essentially undetectable through the 120 hour timepoint.

10 To further support the array data, a panel of cDNA probes and a set of PCR primers for each of the named genes were employed in Northern and RT-PCR analyses, respectively, using an aliquot of RNA reserved from the array experiments (Fig. 8A; data not shown). The  
15 saturation of the PCR reaction was controlled by sampling each reaction together with a  $\beta$ -actin control reaction at different numbers of PCR cycles. All seven genes were differently expressed, as expected from our microarray screen (Fig. 8). SPRY4A, ARHGEF2 (Rho/Rac guanine  
20 exchange factor), RTP801, FLJ20898, and FZD8 (Frizzled Homolog 8) mRNA levels were diminished significantly within 6 h after addition of imatinib, and the level of PDE2A (phosphodiesterase 2A) mRNA decreased measurably 12 h after drug treatment. In contrast, MAFbx levels were  
25 dramatically induced within 6 h of imatinib exposure and remained elevated throughout the treatment (Fig. 8A).

The panel of imatinib-responsive genes was also tested for dependency on ERK1/2 activation by MEK1. Expression patterns of SPRY4A, MAFbx, and ARHGEF2 in  
30 U0126 treated cells were similar to those after imatinib treatment (Figs. 8B and 8C). PDE2A and RTP801 mRNA levels were decreased transiently, and the levels of FLJ20898 and FZD8 did not appear to change noticeably

(Fig. 8B). The level of expression after addition of U0126 was comparable with that seen with imatinib, except that the duration of suppression with a single dose of imatinib was more prolonged (data not shown).

5       The effect of LY294002, a PI3K inhibitor (Vlahos, C.J., et al. (1994) J. Biol. Chem. 269:5214-5248) was also evaluated. Little or no effect on expression levels was observed with any of the imatinib-responsive genes in the restricted panel (Fig. 5D; data not shown). These  
10 results suggest that several of these genes are likely to be dependent on activation of the MAPK signaling pathway by c-KIT, whereas others may not be directly downstream of either MAPK or AKT signaling pathways.

          Additionally, the effects of imatinib on the GRB-  
15 associated docking proteins (GAB) were studied. GAB has been shown to be an adaptor protein capable of binding to many different RTKs (e.g., EGFR, insulin receptor, and cytokine receptor) and other signaling proteins (e.g., GRB2, phospholipase C $\gamma$ , SHP2, PIK3, Shc, and Crk; see,  
20 for example, Holgado-Madruga, M., et al. (1996) Nature 379:560-564; Lehr, S., et al. (1999) J. Biol. Chem. 276:12257-12265). Western blot analysis of lysates from GIST882 cells treated with imatinib demonstrated that while the protein levels of GAB1 remained steady through  
25 the treatment, the amount of phosphorylated GAB1 decreased significantly after 30 minutes (Fig. 9A). Notably, GAB2 protein levels and phosphorylation state did not change with treatment (Fig. 9B). These data reveal that the efficacy of imatinib treatment may also  
30 be correlated with alterations in post-translational modification of different target proteins.

While certain embodiments of the present invention have been described and/or specifically exemplified above, various other embodiments will be apparent to those skilled in the art from the foregoing disclosure.

- 5 The present invention is, therefore, not limited to such embodiments, but is capable of considerable variation and modification without departing from the scope of the following claims.

What is claimed is:

1. A method of assessing the biological activity of a KIT tyrosine kinase inhibitor against a tumor, which  
5 method comprises detecting in a biological sample of said tumor the level of expression of a gene the expression level of which correlates with the biological activity of said KIT tyrosine kinase inhibitor, said biological  
10 sample having been exposed to said KIT tyrosine kinase inhibitor.
2. The method of claim 1, wherein the biological sample is a gastrointestinal stromal tumor.
- 15 3. The method of claim 1, wherein the KIT tyrosine kinase inhibitor is imatinib or a pharmaceutically acceptable salt thereof.
4. The method according to claim 1, which comprises  
20 detecting the level of expression of a nucleic acid sequence provided in Table 2.
5. The method according to claim 1, which comprises detecting the level of expression of a nucleic acid  
25 sequence provided in Table 3.
6. The method according to claim 1, which comprises detecting the level of expression of a nucleic acid  
sequence provided in Table 4.  
30
7. The method according to claim 1, which comprises detecting the level of expression of a nucleic acid sequence encoding Sprouty4A protein.

8. The method according to claim 7, wherein the level of expression of the Sprouty4A gene is detected using an antibody that specifically binds to the Sprouty4A protein.

5

9. The method according to claim 7, wherein the level of expression of the Sprouty4A gene is detected using a nucleic acid probe or primer which specifically hybridizes to the nucleic acid encoding Sprouty4A protein.

10

10. The method according to claim 1, wherein the biological sample has been obtained from a mammal to which the KIT tyrosine kinase inhibitor had been administered.

15

11. The method according to claim 10, wherein said mammal is a human.

20

12. A method of screening a compound for activity against a tumor, which method comprises contacting mammalian tumor cells with the compound and detecting in said tumor cells an altered level of expression of a nucleic acid listed in at least one table selected from the group consisting of Table 2, Table 3 and Table 4 relative to a control.

25

13. The method according to claim 12, which comprises detecting the level of expression of a nucleic acid sequence provided in Table 2.

30

14. The method according to claim 12, wherein expression levels of a nucleic acid sequence listed in Table 3 are decreased by said compound.

5        15. The method according to claim 12, which comprises detecting the level of expression of a nucleic acid sequence provided in Table 4.

10       16. The method as claimed in claim 12, wherein said nucleic acid encodes Sprouty4A.

17. The method of claim 12, wherein the mammalian tumor cells are gastrointestinal stromal tumor cells.

15

18. The method of claim 17, wherein the mammalian tumor cells are a gastrointestinal stromal tumor cell line.

20       19. The method of claim 18, wherein the gastrointestinal stromal tumor cell line is the GIST882 cell line.

20. A method for determining the efficacy of an  
25 anticancer treatment comprising detection of an alteration in expression levels of a biomarker comprising a nucleic sequence provided in a table selected from the group consisting of Table 2, Table 3, and Table 4.

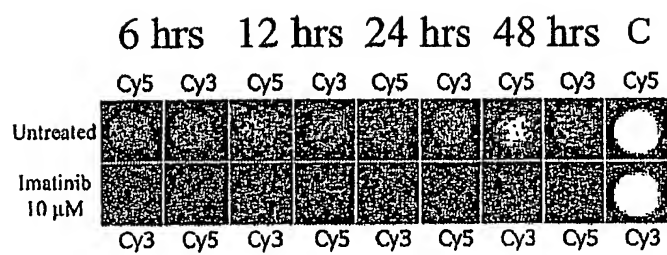
30       21. The method of claim 20, wherein said biomarker is sprouty 4 and said expression level is decreased in response to effective anticancer treatment.

22. The method of claim 20, wherein the biomarker is MAFbx and said expression level is elevated in response to effective anticancer treatment.

5        23. A method for determining the efficacy of an anticancer treatment comprising detection of an alteration in post-translation modification of a biomarker comprising a nucleic sequence provided in a table selected from the group consisting of Table 2,  
10 Table 3, and Table 4.

24. The method as claimed in claim 23, wherein said biomarker is GAB1, and said post translational modification is phosphorylation which is decreased in  
15 response to effective anti-cancer treatment.



**Figure 1**

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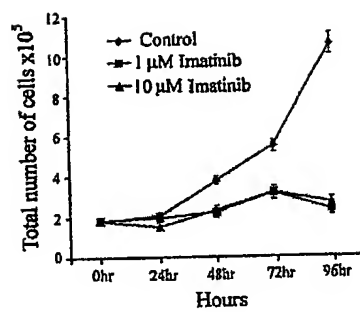


Fig. 2A

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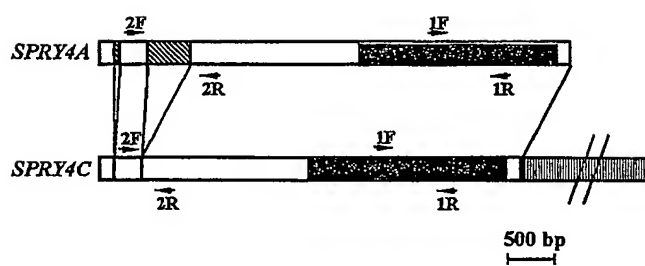


Fig. 2B

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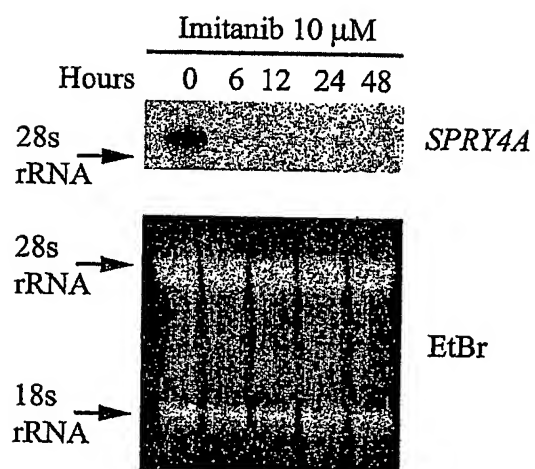
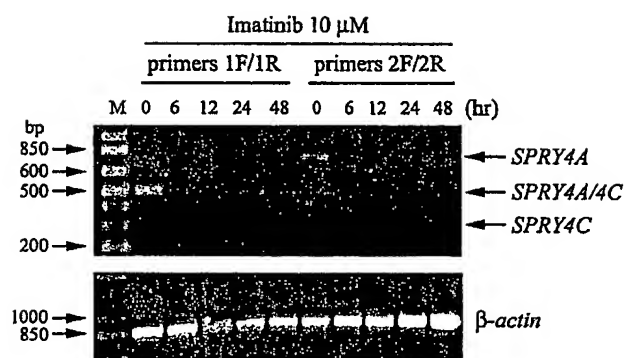


Fig. 2C



**Fig. 2D**

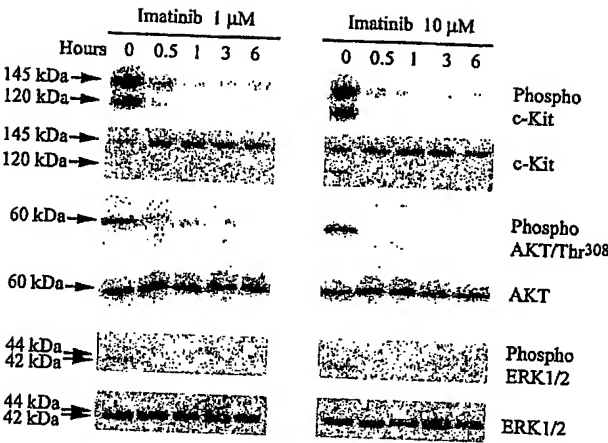


Fig. 3A

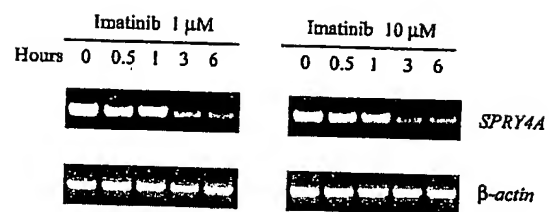


Fig. 3B

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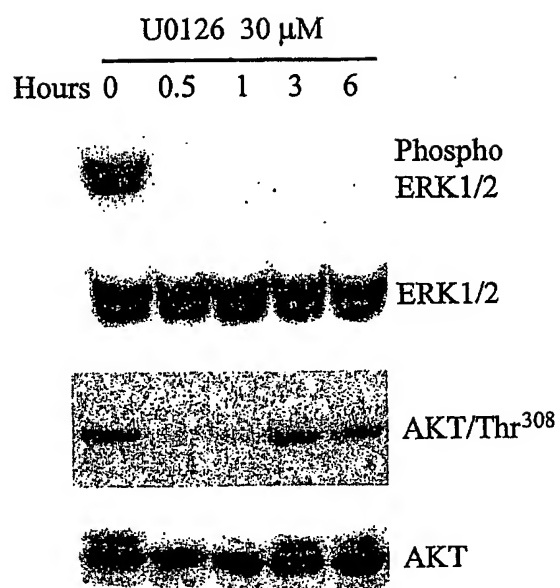


Fig. 4A



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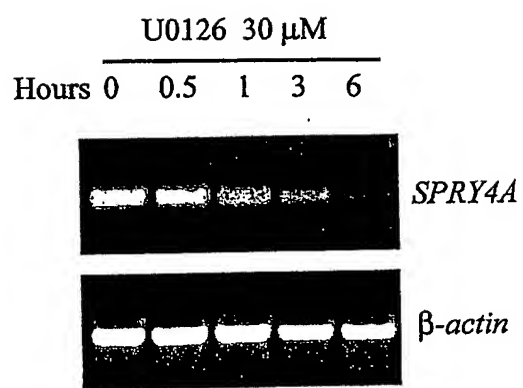


Fig. 4B

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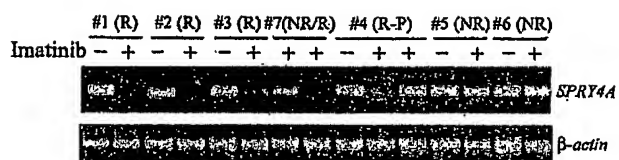


Fig. 5A

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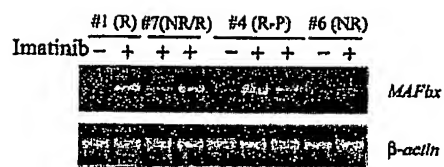


Fig. 5B

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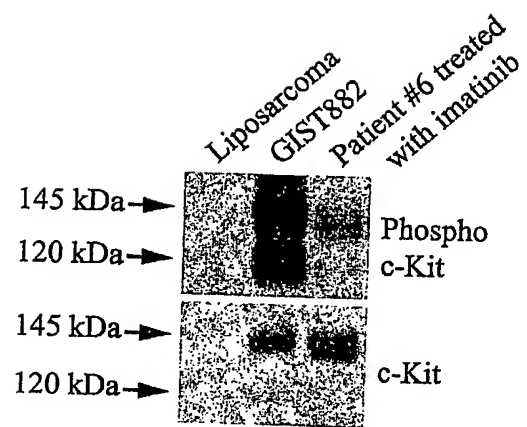


Fig. 6A

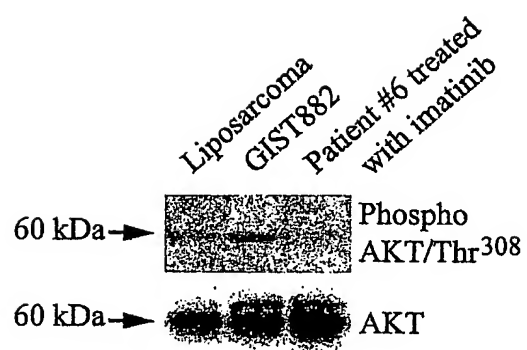


Fig. 6B

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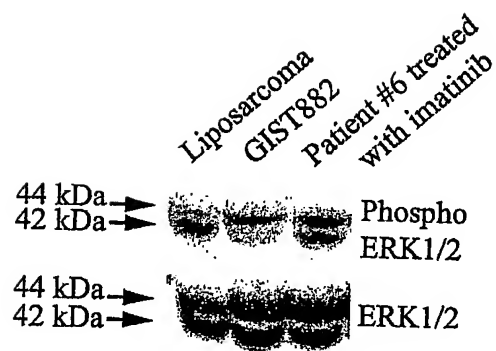


Fig. 6C

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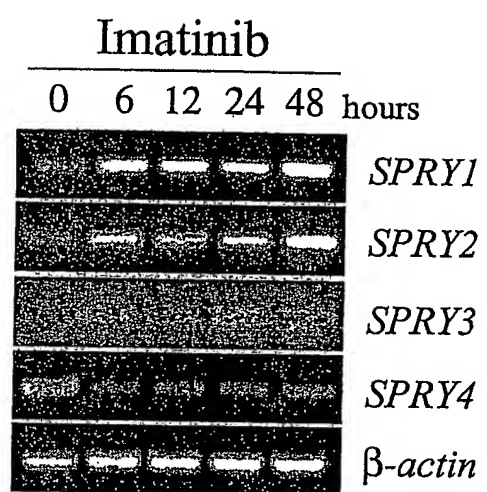
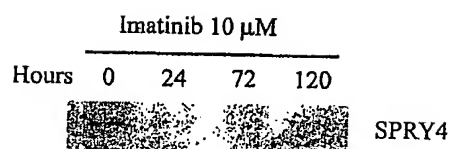


Fig. 7A



**Fig. 7B**



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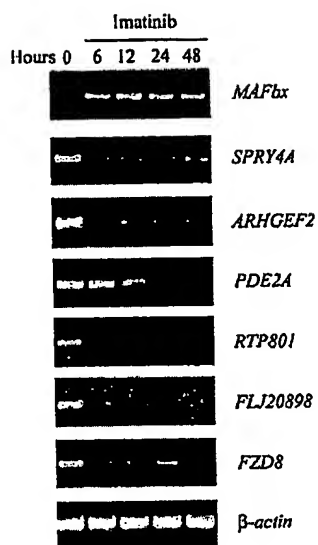


Fig. 8A

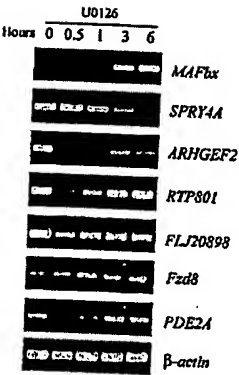


Fig. 8B

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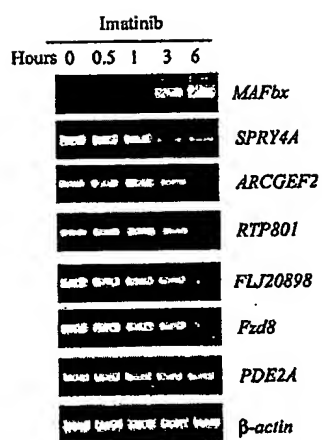


Fig. 8C

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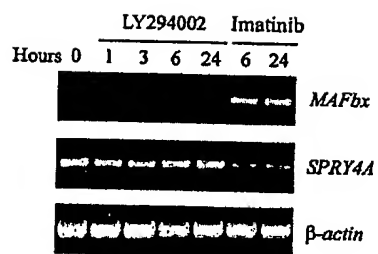


Fig. 8D

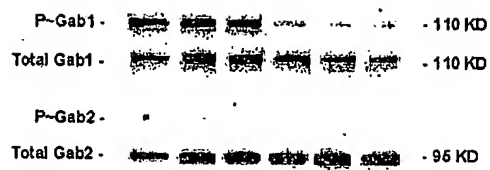


Figure 9

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX — A	FROLOV et al. Response markers and the molecular mechanisms of action of gleevec in gastrointestinal stromal tumors. Molecular Cancer Therapeutics. August 2003, vol. 2, pages 699-709; see Abstract, page 701, column 1, PCR section; page 703, Table 2; page 704, column 1, first complete sentence, Evaluation...section; page 705, Figures A-D; page 706, Figure 7A and 7B; and page 708.	1-3, 6-12, 15-18, 20-24 4, 5, 13, 14 and 19
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